The recognition sequence of site-specific endonuclease *Bbv*II from *Bacillus brevis* 80

N.I. Matvienko, D.M. Pachkunov+ and V.M. Kramarov+

Institute of Protein Research, Academy of Sciences of the USSR, Pushchino, Moscow Region, and +*Institute of Applied Microbiology, Serpukhov, Moscow Region, USSR*

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Site-specific endonuclease *Bbv*II from *Bacillus brevis* 80 recognizes the non-symmetrical hexanucleotide and cleaves DNA at distances of 2 and 6 nucleotides from the recognition site:

5′-GAAGAC2N↓

3′-CTTCTG6N↑

This enzyme may be used in molecular cloning for vectors with multiple restriction sites.

Bacillus brevis *Site-specific endonuclease* *Molecular cloning*

1. INTRODUCTION

More than 400 site-specific endonucleases with 91 different specificities are now known [1]. However, the search for new site-specific endonucleases is continuing as the discovery of enzymes with a new specificity facilitates DNA sequencing and DNA cloning. Among the known restrictases, a few recognize non-symmetrical sequences [1]. The new site-specific endonuclease *Bbv*II isolated by us from *Bacillus brevis* 80 belongs to this type [2]. It recognizes the non-symmetrical hexanucleotide and cleaves DNA at distances of 2 and 6 nucleotides from the recognition site:

5′-GAAGAC2N↓

3′-CTTCTG6N↑

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

B. brevis 80 has been isolated by us and identified by Z.F. Bunina on the basis of the usual taxonomical criteria [2].

2.2. *Enzymes*

Endonuclease *Eco*RI was isolated as in [3], and *Bme*I as in [4]. DNA-polymerase I (Klenow fragment) and nuclease P1 were obtained from Boehringer-Mannheim and *Escherichia coli* alkaline phosphatase from Sigma (USA). Polynucleotide kinase was a gift of M.I. Boleznin (Institute of Applied Microbiology).

2.3. *DNA and reagents*

Plasmid pBR322 was isolated from the *E. coli* RR1 strain as in [5] and then additionally purified on a column with Biogel A50 (Bio-Rad, USA). Heparin-agarose was prepared as in [6]. [γ-^{32}P]ATP (>3000 Ci/mmol) and [α-^{32}P]ATP (>2000 Ci/mmol) were obtained from Amersham (England).

2.4. *Isolation of* Bbv*II*

Endonuclease *Bbv*II (2000 units) was partially purified from 3 g cell paste as in [2].

2.5. *DNA sequencing*

Determination of cleavage points on DNA produced by endonuclease *Bbv*II was carried out as in [7] with a few modifications. Instead of ethanol precipitation of DNA we used the DEAE-cellulose column technique, as in [8].

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2.6. *Determination of the end nucleotides after digestion of pBR322 DNA with* Bbv*II*

To determine the 5′-terminal nucleotides, the pBR322 DNA was hydrolyzed with endonuclease *Bbv*II which recognizes on the plasmid sites with coordinates 739, 1602 and 4355 nucleotides [2], and yielded after hydrolysis fragments with protruding 5′-termini. The fragments were dephosphorylated and 5′-labeled using [γ-^{32}P] and T4 polynucleotide kinase. The mixture of ^{32}P-labeled fragments was cleaved additionally with endonuclease *Sal*I and separated on an 8% polyacrylamide gel. The smallest labeled fragment B (fig.1) with the coordinates of the sites 653–739 was eluted from the gel. In another case the labeled fragments were cleaved with *Bme*I and separated on an 8% polyacrylamide gel. As expected, this resulted in 5 labeled fragments. Three of them with coordinates 739–801 (fragment C), 1482–1602 (D) and 1602–1762 (E) (fig.1) were eluted from the gel.

All the isolated fragments were digested to completion with nuclease P1 from *Penicillum citrinum*. The reaction mixtures were separated by electrophoresis on Whatman 3 MM paper in puridine-acetate–EDTA buffer (pH 3.5) at 50 V/cm. A mixture of 4 unlabeled deoxymononucleotides, 10 μg each (Sigma) were added as carriers to achieve optical visibility.

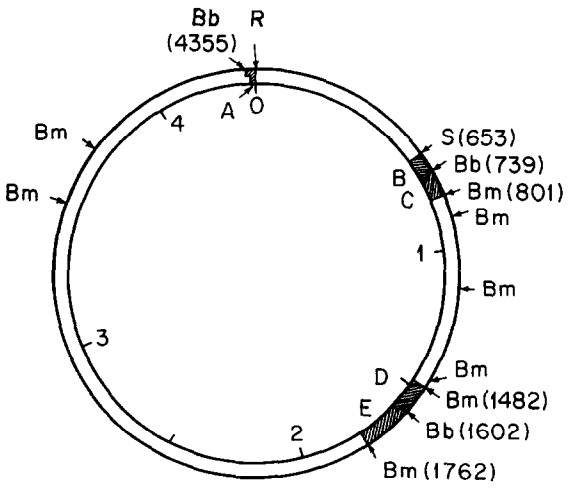

Fig.1. Localization of DNA fragments used for determination of pBR322 cleavage points with *Bbv*II. The fragments used for sequencing (A) and for determination of end nucleotides (B–E) are hatched. The numbers in brackets are the coordinates of the recognition site centers based on the corrected nucleotide sequence of pBR322 [12]. Bm, *Bme*I; Bb, *Bbv*II; S, *Sal*I.

2.7. *Determination of homogenicity of the ends of the DNA fragments after digestion with* Bbv*II*

To determine homogenicity of the ends of the fragments, 4 mononucleotide spots observed were cut off under UV-light and used directly for measuring their ^{32}P-activities in a liquid toluene scintillator.

3. RESULTS AND DISCUSSION

Endonuclease *Bbv*II was isolated from the crude extract of *B. brevis* 80 by chromatography on Ultragel AcA 34 and heparin-agarose. The partially purified enzyme did not cut away the end phosphate of DNA and was used in sequence analysis of the DNA fragments produced by this endonuclease by the method in [7] to determine the points of cleavage of DNA with this enzyme.

Computer treatment on the basis of DNA cleavage data with known nucleotide sequences (pBR322, ϕX174) has shown that *Bbv*II recognizes the nucleotide sequence 5′-GAAGAC-3′ on double-stranded DNA (only one strand is given). This sequence is located on pBR322 DNA at the coordinates of 739, 1602 and 4355 nucleotide base pairs [2]. The fragment A of pBR322 DNA obtained by the action of *Eco*RI (0 point) and *Bbv*II on the site with coordinates of 4355 nucleotides was used for determination of cleavage points of DNA by endonuclease *Bbv*II (fig.1). Firstly, pBR322 was digested with *Eco*RI, then the 5′-termini were labeled with [γ-^{32}P]ATP and polynucleotide kinase and the labeled DNA was digested with *Bbv*II. The digestion products were separated on a 15% polyacrylamide gel. The smaller one was eluted from the gel and the nucleotide sequence of the end opposite the labeled one was determined. The sequence 5′...CTT*GAAGAC*GA-3′ was found at the 3′-end of fragment A. The recognition sequence is underlined.

To find the second point of cleavage near this recognition site, the 3′-ends of pBR322 DNA after cleavage with *Eco*RI endonuclease were labeled using the Klenow fragment of DNA polymerase I of *E. coli* and [α-^{32}P]dATP. Then the labeled DNA was cleaved with *Bbv*II. As in the first case, the

fragments were separated on polyacrylamide gel and the smaller one was eluted. The nucleotide sequence of the 5′-end was shown to be 5′-CCTTTC*GTCTTC*A...-3′.

Using high-voltage electrophoresis on paper the end nucleotides of the fragments produced by *Bbv*II at cleavage near the sites with coordinates 739 and 1602 were determined (fig.2). It was found that the end-labeled nucleotides of the B, C, D, E fragments are T, A, T and T, respectively.

It is concluded that after hydrolysis of DNA with *Bbv*II the ends of the produced fragments have the structure:

5′-GAAGAC $N_1^{'}N_2^{'}$-3′
3′-CTTCTG $N_1N_2N_3N_4N_5N_6$-5′

(N_x, any nucleotide, $N_x^{'}$, complementary to it).

To determine homogeneity of the ends of the produced fragments and fitness of the obtained *Bbv*II preparations for construction of recombinant DNA and sequencing, the spots on the paper corresponding to all the four nucleotides were cut out and counted in a scintillation counter. The label distribution is presented in table 1. The result

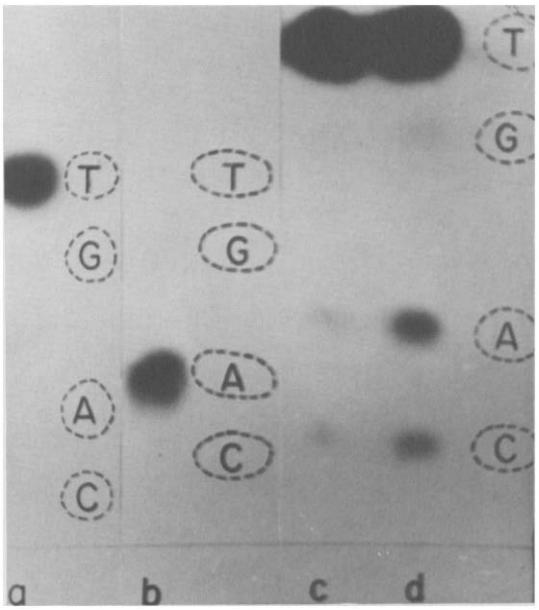

Fig.2. High-voltage paper electrophoresis of labeled end nucleotides (1500 V, 75 mA, Whatman 3 MM 30 × 15 cm paper). (a) B fragment, electrophoretic run 35 min; (b) C fragment, electrophoretic run 40 min; (c,d) D,E fragments, electrophoretic run 55 min.

Table 1

Label distribution in the end nucleotides of DNA fragments of pBR322 produced by *Bbv*II (%)

Nucleotide	Fragment			
	B	C	D	E
A	0.1	100	0.0	6.4
T	99.7	0	92.3	82.5
G	0.2	0	7.7	5.5
C	0.0	0	0.0	5.6

of this experiment shows that the *Bbv*II endonuclease isolated by the described method is really fit for cloning and sequencing.

As the DNA fragments produced by this endonuclease have unique protruding tetranucleotide termini, the DNA ligase must join them in an initial order as has been shown for restrictase *Hga*I [9]. This property of *Bbv*II may be used for insertion of a foreign fragment of DNA in vector molecules having multiple cleavage sites (fig.3). Firstly, the vector should be cleaved with *Bbv*II and a fragment containing one site, *x*, for insertion of foreign fragment should be isolated. Then the fragment should be cleaved with endonuclease X and the obtained arms should be joined with the foreign fragment by DNA ligase. Next, the products of ligase reaction should be mixed with the original *Bbv*II fragments of the vector and ligated again. After this procedure part of the molecules will be a vector with the integrated foreign fragment.

*Bbv*II may be also successfully used to facilitate sequencing of DNA fragments inserted in *Hin*dIII

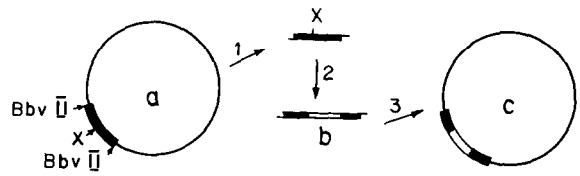

Fig.3. Integration of a foreign fragment in a multisite vector. (a) A multisite vector for endonuclease X; (b) a foreign fragment; (c) recombinant DNA. (1) Vector cleavage with *Bbv*II, isolation of individual fragment. (2) Insertion of a foreign fragment in cleavage site *x*. (3) Ligation of the recombinant fragment with the *Bbv*II fragments of the original vector.

and *Cla*I sites of pBR322 as was proposed for the pUR222 and pUR250 vectors [10,11]. A label should be introduced at the *Eco*RI termini and then the recombinant DNA should be cleaved with *Bbv*II. The smaller labeled fragment will not interfere because its length is less than the distances between *Eco*RI and *Cla*I or the *Eco*RI and *Hin*dIII sites.

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